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Asymmetric Hsp90 N Domain SUMOylation Recruits Aha1 and ATP-Competitive Inhibitors

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SUMMARY

The stability and activity of numerous signaling proteins in both normal and cancer cells depends on the dimeric molecular chaperone heat shock protein 90 (Hsp90). Hsp90's function is coupled to ATP binding and hydrolysis and requires a series of conformational changes that are regulated by cochaperones and numerous posttranslational modifications (PTMs). SUMOylation is one of the least-understood Hsp90 PTMs. Here, we show that asymmetric SUMOylation of a conserved lysine residue in the N domain of both yeast (K178) and human (K191) Hsp90 facilitates both recruitment of the adenosine triphosphatase (ATPase)-activating cochaperone Aha1 and, unexpectedly, the binding of Hsp90 inhibitors, suggesting that these drugs associate preferentially with Hsp90 proteins that are actively engaged in the chaperone cycle. Importantly, cellular transformation is accompanied by elevated steady-state N domain SUMOylation, and increased Hsp90 SUMOylation sensitizes yeast and mammalian cells to Hsp90 inhibitors, providing a mechanism to explain the sensitivity of cancer cells to these drugs.

INTRODUCTION

The evolutionarily conserved molecular chaperone heat shock protein 90 (Hsp90) is an essential component of the cellular homeostatic machinery in eukaryotes (Picard, 2002; Taipale et al., 2010). Cancer cells strongly depend on Hsp90 because of their need to cope with constitutive genetic instability and

frequent environmental insults, including nutrient deprivation, hypoxia, and proteotoxic stress (Treipel et al., 2010), and emerging clinical data identify Hsp90 inhibition as a promising therapeutic strategy to treat cancer (Neckers and Workman, 2012). Cancer cells appear to be particularly sensitive to Hsp90 inhibitors when compared to nontransformed cells (Bisht et al., 2003), and these drugs are retained by tumors in vivo far longer than in normal tissues (Treipel et al., 2010). However, the molecular basis for these phenomena remains undefined.

The Hsp90 molecule is a dimer, and each protomer can be divided into structurally and functionally distinct domains: (i) an N-terminal domain that contains an ATP-binding pocket, which can also be occupied by small molecule inhibitors, as well as sites for cochaperone and client binding; (ii) a middle domain that provides additional cochaperone and client-interacting surfaces and also completes the split adenosine triphosphatase (ATPase) motif of Hsp90; and (iii) a C-terminal domain providing sites for constitutive dimerization and for cochaperone and client interaction (Ali et al., 2006; Shiao et al., 2006; Stebbins et al., 1997). Hsp90 chaperone function depends on an ordered sequence of dynamic conformational changes that are linked to binding and hydrolysis of ATP and are disrupted by drug occupancy of the ATP pocket (Hessling et al., 2009; Mayer, 2010; McClellan et al., 2007; Picard, 2002; Prodromou, 2012; Treipel et al., 2010). In eukaryotes, the Hsp90 conformational cycle is facilitated by a number of proteins, termed cochaperones, that interact with distinct Hsp90 conformations and serve discrete functions (Cox and Johnson, 2011; Li et al., 2012). Growing evidence suggests that Hsp90 interacts asymmetrically with clients and some cochaperones and that each Hsp90 protomer can hydrolyze ATP independently (Cunningham et al., 2008; Vaughan et al., 2006). A number of cochaperones (e.g., p50/Cdc37, HOP/Sti1, p23, Aha1) modulate the rate of Hsp90-mediated ATP hydrolysis, thereby regulating the rate of the chaperone cycle and tailoring Hsp90 chaperone activity to the

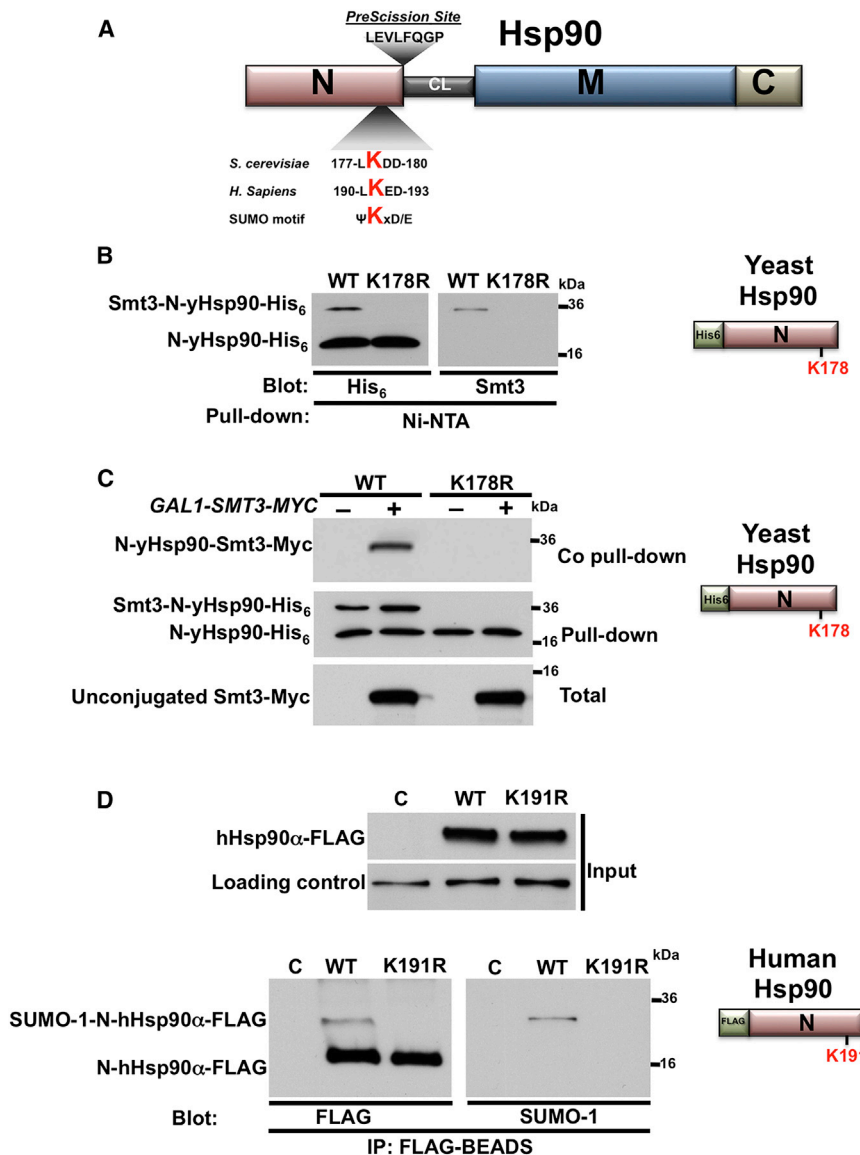


Figure 1. The N Domain of Yeast and Human Hsp90 Is SUMOylated In Vivo

(A) Schematic representation of yHsp90 and hHsp90 α showing the putative N domain SUMO consensus motif. Proposed SUMO modification sites are in red.

(B) N domain SUMOylation of wild-type (WT) and yHsp90-K178R (K178R) was detected by immunoblotting with anti-hexahistidine and anti-Smt3 after PreScission Protease digestion.

(C) Yeast cells expressing wild-type (WT) or yHsp90-K178R (K178R) and carrying SMT3-MYC under GAL1 promoter were grown on raffinose (–) or galactose (+). Hsp90 modification by Smt3-Myc was assessed by immunoblotting Hsp90 pull-downs.

(D) SUMOylation of N domain wild-type (WT) FLAG-hHsp90 α and K191R mutant. Hsp90 proteins were immunoprecipitated, subjected to PreScission Protease digestion, and immunoblotted with anti-FLAG and anti-SUMO-1. Lysates from cells transfected with empty plasmid (C) were used as a control. See also Figure S1.

covalently linked to lysine residues in a process similar to ubiquitination and, in doing so, affect the conformation, protein-protein interaction, or subcellular location of the modified protein (Hickey et al., 2012; Ulrich, 2009). Although SUMOylation of yeast Hsp82 (yHsp90) and human Hsp90 α (hHsp90 α) has been reported previously (Panse et al., 2004; Pountney et al., 2008; Zhou et al., 2004), identification of individual SUMOylated lysine residues and their impact on Hsp90 function remains unexplored. Here, we report that SUMOylation of an N domain lysine conserved in both yeast and human Hsp90 initiates recruitment of Aha1 to the chaperone complex in cells. Unexpectedly, N domain

SUMOylation also facilitates binding of Hsp90 inhibitors and sensitizes yeast and mammalian cells to these drugs.

RESULTS

Identification of a SUMOylated Lysine in the Hsp90 N Domain

We identified a conserved lysine residue (K178) in the N domain of yeast Hsp82 (yHsp90) that is within the SUMO recognition motif Ψ -K-x-D/E (Ψ is a hydrophobic amino acid and x is any amino acid) (Sampson et al., 2001) (Figure 1A). K178, in the N-terminal domain (open ATP lid), is situated in a solvent-exposed loop and thus is potentially susceptible to posttranslational modification (Protein Data Bank [PDB] ID code 1AMW), although the side-chain amine of K178 makes a series of interactions with the main-chain carbonyl groups of I20, F128, and L129. In the closed state (PDB: 2CG9), however, K178 becomes buried in the N-terminal

needs of specific clients (Panaretou et al., 2002; Retzlaff et al., 2010; Siligardi et al., 2004). Importantly, Aha1, by facilitating energy-intensive conformational changes necessary to establish Hsp90 ATPase competence, markedly stimulates the weak endogenous ATPase activity of Hsp90 and is thus considered to be a crucial component of active Hsp90 chaperone complexes (Li et al., 2013; Lotz et al., 2003; Panaretou et al., 1998).

In *Saccharomyces cerevisiae*, the abundance of Hsp90 far exceeds that of its cochaperones (<http://yeastgfp.yeastgenome.org/>). Thus, mechanisms must exist to facilitate the controlled interaction of these proteins at sites of chaperone activity. Eukaryotic Hsp90 is subject to numerous posttranslational modifications (PTMs) that play significant roles in regulating association and dissociation of specific cochaperones (Mollapour and Neckers, 2012; Muller et al., 2013; Soroka et al., 2012; Walton-Diaz et al., 2013; Xu et al., 2012). SUMOylation is a reversible PTM in which small ubiquitin-like modifier (SUMO) proteins are

dimerization interface, and the side-chain amine of K178 becomes hydrogen bonded to one of the two carboxyl side-chain oxygens of D132 and to the main-chain carbonyl oxygens of A131 and F128. Thus, posttranslational modification of K178 is likely only possible prior to ATP lid closure and N domain dimerization, but modification of this residue also appears to require additional conformational changes not visible in these crystal structures.

To ascertain whether this residue is SUMOylated in cells and to determine if it was the only SUMOylated residue in the yHsp90 N domain, we mutated K178 to nonSUMOylatable arginine. We also introduced a PreScission Protease cleavage site between the N domain and adjacent charged linker region of both wild-type yHsp90 and yHsp90-K178R, allowing us to separate the N domain from the full-length Hsp90 protein (which is predicted to have additional SUMOylation sites in other domains) (Figure 1A). These yHsp90 proteins were hexahistidine epitope-tagged at their N terminus and expressed as the sole copy of yHsp90 in yeast. It is notable that insertion of a PreScission Protease site upstream of the N domain did not interfere with the chaperone activity of wild-type yHsp90 (Figures S1A–S1D available online). The hexahistidine epitope tag allowed us to isolate Hsp90 proteins from cell lysates using Ni-NTA agarose beads. We then treated the pull-downs with PreScission Protease to isolate yHsp90 N domains. Western blotting of the isolated N domains with antibodies to both hexahistidine and Smt3 (the only SUMO protein in yeast) confirmed a slower migrating band to be SUMOylated yHsp90 (Figure 1B). These data also confirmed that K178 is the only SUMOylated lysine in the yHsp90 N domain (Figure 1B). Lysine can also be targeted by other PTMs, including ubiquitination and acetylation; however, our data suggest that K178 is only subject to SUMOylation (Figures S1E and S1F).

Smt3 is an essential protein in yeast. To query the impact of modulating cellular Smt3 expression on yHsp90 SUMOylation, we overexpressed Myc-epitope-tagged *SMT3* under galactose-inducible promoter (*GAL1*) in yeast expressing either wild-type yHsp90 or yHsp90-K178R. After inducing *SMT3-MYC* expression by growing the cells on galactose media, we could detect Smt3-modified wild-type yHsp90 N domain, but the N domain of yHsp90-K178R remained unmodified (Figure 1C).

To determine whether the corresponding lysine residue (K191) in human Hsp90 α (hHsp90 α) (see Figure 1A) is also subject to SUMOylation, we introduced a PreScission site between the N domain and charged linker of FLAG-tagged wild-type hHsp90 α and hHsp90 α -K191R. After verifying equivalent expression of both proteins in transiently transfected human embryonic kidney 293 (HEK293) cells (Figure 1D), we confirmed SUMOylation of K191 in the hHsp90 α N domain using anti-FLAG and anti-SUMO-1 (Figure 1D). Mammalian cells express three SUMO proteins: SUMO-1, SUMO-2, and SUMO-3. Reprobing the blot in Figure 1D with anti-SUMO-2/SUMO-3 did not yield a detectable signal, suggesting that the hHsp90 α N domain is modified only by SUMO-1 (data not shown). Unlike in yeast, K191 is also subject to ubiquitination and acetylation (Figures S1G and S1H).

Hsp90 N Domain SUMOylation Recruits the Cochaperone Aha1

PTMs can facilitate or inhibit interaction of cochaperones with Hsp90 in cells (Mollapour and Neckers, 2012; Xu et al., 2012).

Therefore, we examined the impact of N domain SUMOylation on Hsp90 binding to selected cochaperones. Hsp90 proteins from yeast cells expressing either hexahistidine epitope-tagged wild-type yHsp90 or the nonSUMOylatable K178R mutant were isolated with Ni-NTA agarose, and associating cochaperones were examined by western blot. Interaction of yAha1 with the K178R mutant was abrogated (Figure 2A), as was interaction of nonSUMOylatable hHsp90 α -K191R with human Aha1 (hAha1) (Figure 2B). In contrast, nonSUMOylatable yeast and human Hsp90 proteins readily associated with Cdc37^{p50}, Sti1^{HOP}, and Sba1^{p23} (Figures S2A and S2B). Next, we examined whether overexpression of yeast *SMT3-MYC* or human *SUMO-1-HA* could ameliorate the observed reduction in Aha1 interaction. While overexpression of *SMT3-MYC* increased SUMOylation of wild-type yHsp90 (Figure 2C) and enhanced its interaction with yAha1 (Figures 2D and S2A), it did not restore yAha1 interaction with yHsp90-K178R (Figure 2D). We observed comparable results when *SUMO-1-HA* was coexpressed with either wild-type hHsp90 α or hHsp90 α -K191R in COS7 cells (Figures 2E, 2F, and S2B).

Based on these data, we asked whether Aha1 preferentially interacts with N domain SUMOylated yHsp90. Yeast Aha1-FLAG was expressed under its native promoter and immunoprecipitated from cells expressing either wild-type yHsp90 or yHsp90-K178R. After PreScission Protease cleavage, western blotting for coimmunoprecipitated yHsp90 N domains revealed a doublet of equal intensity corresponding to SUMOylated and nonSUMOylated N domains (Figure 2G). Overexpression of *SMT3-MYC* enhanced the interaction of yAha1 with yHsp90 and proportionately enhanced the intensity of the yHsp90 N domain doublet (Figure 2G). These data, together with the lack of detectable yAha1 binding to yHsp90-K178R, suggest a model in which yAha1 is recruited to asymmetrically SUMOylated yHsp90. Although it is not possible to detect yAha1 interaction with yHsp90-K178R under steady-state conditions (or even when yAha1 is overexpressed using its native promoter), association with yHsp90-K178R is detectable upon strong overexpression of yAha1 (using a *GAL1* promoter on a yeast episomal plasmid; see Supplemental Information) (Figure 2H). This is consistent with binding data obtained using bacterially expressed (thus lacking posttranslational modifications) and purified yAha1 and yHsp90 proteins. In vitro, the affinities of both wild-type yHsp90 and yHsp90-K178R proteins for yAha1 were similar (wild-type yHsp90 $K_D = 0.42 \pm 0.08 \mu\text{M}$; yHsp90-K178R $K_D = 1.1 \pm 0.1 \mu\text{M}$; Figures S2C and S2D, respectively). These data are in agreement with previously published values (Xu et al., 2012). They confirm that K178 mutation does not structurally compromise yHsp90, and they are consistent with the ability of yHsp90-K178R to support yeast viability (Figures S1A–S1D).

Detectable Aha1 Binding to Hsp90 in Cells Requires SUMOylation of One Protomer

Our data suggest that, in cells, yAha1 may interact with yHsp90 in which only one of the protomers is SUMOylated on K178 (Figure 2G). To explore this possibility further, we coexpressed two copies of yHsp90 (with a PreScission cleavage site as in Figure 1A) in yeast, with one copy carrying a hexahistidine (His₆) tag and the other copy carrying a GLU/GLU (GLU) epitope tag.

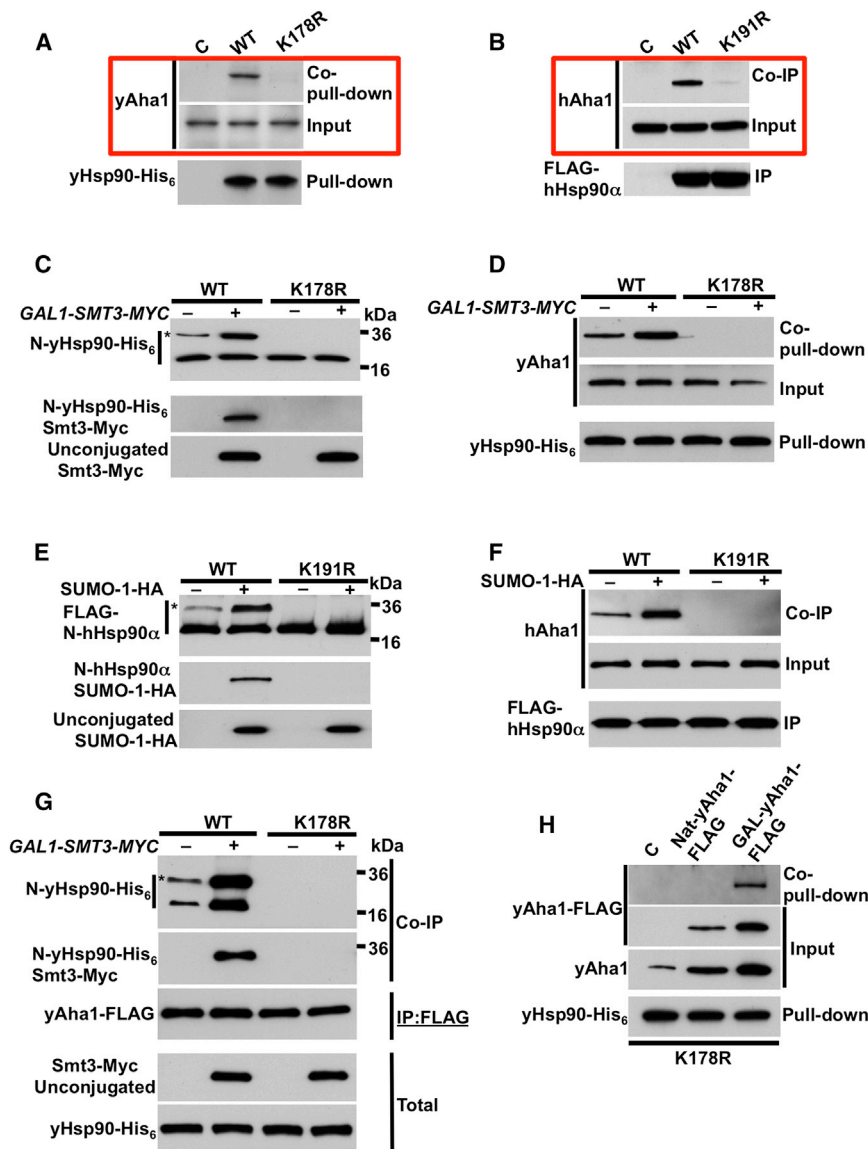


Figure 2. Aha1 Binds to N Domain SUMOylated Hsp90

(A) Protein extracts from control (C), wild-type yHsp90 (WT), and nonSUMOylatable yHsp90-K178R (K178R)-expressing cells were precipitated using Ni-NTA agarose, and yAha1 interaction was detected by immunoblotting.

(B) COS7 cells were transfected with empty plasmid (C), FLAG-hHsp90α (WT), or nonSUMOylatable hHsp90α-K191R constructs. hAha1 interaction with these Hsp90 proteins was examined by immunoprecipitation (IP) and immunoblotting.

(C) Yeast cells expressing wild-type yHsp90 (WT) or yHsp90-K178R (K178R), and carrying GAL1-SMT3-MYC plasmid, were grown on raffinose (-) or galactose (+). N domain modification (*) by Smt3-Myc was assessed following precipitation with Ni-NTA agarose, PreScission Protease digestion, and immunoblotting.

(D) yAha1 interaction with wild-type yHsp90 (WT) or yHsp90-K178R (K178R) from cells overexpressing GAL1-SMT3-MYC and grown on raffinose (-) or galactose (+) was assessed following Ni-NTA agarose pull-down and immunoblotting.

(E) COS7 cells transiently transfected with FLAG-hHsp90α (WT) or nonSUMOylatable hHsp90α-K191R were cotransfected with SUMO-1-HA or empty plasmid. N domain hHsp90α modification (*) by SUMO-1-HA was assessed following IP, PreScission Protease digestion, and immunoblotting.

(F) hAha1 interaction with wild-type or nonSUMOylatable Hsp90 treated as in (E) was examined following IP and immunoblotting.

(G) Yeast cells expressing yHsp90 (WT) or yHsp90-K178R (K178R), and coexpressing yAha1-FLAG and GAL1-SMT3-MYC plasmid, were grown on raffinose (-) or galactose (+). yAha1-FLAG was immunoprecipitated with anti-FLAG-agarose, and yAha1 interaction with SUMOylated (*) and nonSUMOylated yHsp90 was assessed by immunoblotting.

(H) NonSUMOylatable yHsp90-K178R-expressing cells were transformed with empty plasmid (C) or multicopy plasmid expressing yAha1-FLAG under native promoter (Nat) or GAL1 promoter (GAL). yHsp90-His₆ was isolated by Ni-NTA agarose pull-down, and yAha1-FLAG interaction was examined by immunoblotting. See also Figure S2.

The cells also overexpressed SMT3-MYC. First, we verified equal expression of both epitope-tagged yHsp90 proteins (Figure 3A, top two panels). Next, we isolated yHsp90-His₆ using Ni-NTA agarose, followed by elution with imidazole and immunoprecipitation with antibody recognizing the GLU/GLU epitope tag (Figure 3A, middle panel). Immobilized yHsp90-His₆/yHsp90-GLU (WT_{His6}/WT_{GLU}) heterodimers were then treated with PreScission Protease in order to isolate the yHsp90 N domains (Figure 3A, cartoon of flow scheme). Similar experiments were conducted with yeast expressing combinations of wild-type and K178R yHsp90 or yHsp90-K178R only (e.g., WT_{His6}/K178R_{GLU}; WT_{GLU}/K178R_{His6}; K178R_{His6}/K178R_{GLU}). After protease cleavage, western blotting with anti-His₆ or anti-

GLU/GLU identified a doublet of equal intensity for WT_{His6}/WT_{GLU} samples (Figure 3A, bottom two panels, first lane). In the WT_{His6}/K178R_{GLU} and K178R_{His6}/WT_{GLU} samples, the tag antibody recognizing the wild-type protomer also identified a doublet of equal intensity, while the tag antibody recognizing the mutated protomer identified only a single band whose molecular weight is consistent with lack of SUMOylation (Figure 3A, bottom two panels, lanes 2 and 3). Finally, when yHsp90 was comprised of two untagged protomers, antibodies recognizing either tag identified a single unmodified species (Figure 3A, bottom two panels, lane 4).

These data suggest that a nonSUMOylated yHsp90 protomer readily dimerizes with either another nonSUMOylated protomer

or a SUMOylated protomer (Figure 3A, schematic diagram). To explore the possibility that two SUMOylated protomers might dimerize, we immunoprecipitated Smt3-Myc from yeast lysates expressing wild-type yHsp90His₆. The immobilized yHsp90 proteins were treated with PreScission Protease, and isolated N domains were visualized by western blot. If two SUMOylated protomers dimerized, we would expect the abundance of the slower-migrating (SUMOylated) N domain to be greater than that of the faster migrating (nonSUMOylated) species. However, we always observed a doublet of nearly equal intensity upon probing with anti-His₆ (Figure 3B, upper panel). Probing the same blot with anti-Myc confirmed that the slower-migrating band reflected SUMOylated yHsp90 (Figure 3B, middle panel).

These findings prompted us to reexamine yAha1 binding to SUMOylated yHsp90. We immunoprecipitated FLAG-yAha1 from yeast cells overexpressing *SMT3-MYC* followed by PreScission Protease treatment to isolate N domains of the coimmunoprecipitated His₆- or GLU-tagged yHsp90 proteins. We verified that yAha1 does not bind to yHsp90 comprised of two nonSUMOylated protomers (Figure 3C, lane 4), and we confirmed that the cochaperone binds with equal efficiency to a yHsp90 molecule comprised of either one wild-type and one K178R protomer (Figure 3C, lanes 2 and 3) or two wild-type protomers (Figure 3C, lane 1). These data provide further evidence that SUMOylation of one Hsp90 protomer is necessary and sufficient for optimal yAha1 recruitment in yeast cells.

To determine whether ATP-binding affects Hsp90 N domain SUMOylation, we examined two well-characterized yHsp90 mutants: E33A, which efficiently binds, but poorly hydrolyzes, ATP (the equivalent hHsp90 mutant displays increased Aha1 association in cells; Xu et al., 2012), and D79N, which does not bind ATP (Obermann et al., 1998; Panaretou et al., 1998). We expressed both hexahistidine-tagged mutants and wild-type yHsp90 in yeast cells, and we examined their steady-state N domain SUMOylation after affinity pull-down and PreScission cleavage. N domain SUMOylation of yHsp90-E33A was markedly greater than that of wild-type yHsp90. In contrast, N domain SUMOylation of yHsp90-D79N was not detectable (Figure 3D). These data suggest that ATP binding to Hsp90 is an important prerequisite for N domain SUMOylation, consistent with the requirements for optimal Aha1 association. This model predicts that N domain deSUMOylation occurs prior to initiation of a new chaperone cycle.

Loss of Hsp90 N Domain SUMOylation Minimally Affects Several Hsp90-Dependent Clients

Protein SUMOylation impacts numerous biological pathways, including nuclear-cytoplasmic transport, cell cycle control, and stress responses (Johnson, 2004; Ulrich, 2009). However, we found that neither α factor-mediated G1 arrest nor heat shock caused an increase in steady-state yHsp90 N domain SUMOylation (Figure S3A), and yeast expressing only yHsp90-K178R mounted a normal transcriptional response to heat shock (Figure 4A). Likewise, yeast cells expressing yHsp90-K178R displayed no growth or cell cycle defects (Figures S3B–S3D), nor did this mutation affect nuclear or cytoplasmic distribution of yHsp90-GFP (data not shown).

When we queried the ability of yHsp90-K178R or hHsp90 α -K191R to interact with and chaperone several Hsp90 clients previously reported to require Aha1/Hsp90 association (Lotz et al., 2003; Panaretou et al., 2002), we found that this mutation failed to affect Hsp90-dependent chaperoning of the glucocorticoid receptor (GR) or the active form of Ste11 kinase (Ste11 Δ N), the ortholog of mammalian Raf-1 (Flom et al., 2008; Louvion et al., 1998) (Figures 4B and 4C). Similarly, hHsp90 α -K191R associated with GR and Raf-1 equivalently to wild-type hHsp90 α (Figure 4D). NonSUMOylatable yeast and human Hsp90 proteins also interacted with and chaperoned v-Src with efficiency equal to that of wild-type Hsp90 (Figures 4E and 4F). These data suggest that the attenuated interaction of yHsp90-K178R with yAha1 does not phenocopy *yaha1* deletion (Lotz et al., 2003; Panaretou et al., 2002). This was further supported by comparing the growth of yeast expressing either yHsp90-K178R or wild-type yHsp90 but lacking yAha1 expression on glucose (YPDA) and respiratory (YPEG) growth media at an elevated temperature. As previously reported (Lotz et al., 2003; Panaretou et al., 1998), *yaha1 Δ* cells displayed a temperature sensitivity phenotype on YPDA and YPEG media (Figure S3E). In contrast, yeast expressing yHsp90-K178R displayed no growth defects on either media, consistent with the possibility that Aha1 may have Hsp90-independent activity (Sun et al., 2012).

Aha1 stimulates Hsp90-mediated ATP hydrolysis and thus reduces the dwell time of clients in the chaperone complex, which has deleterious consequences for difficult-to-fold clients that require prolonged association with Hsp90 (such as the cystic fibrosis transmembrane conductance regulator protein, CFTR) (Loo et al., 1998; Youker et al., 2004). We examined the ability of nonSUMOylatable yHsp90 to promote CFTR stabilization and maturation. Yeast cells were cotransfected with HA-tagged CFTR (under the control of a constitutive promoter) and either wild-type yHsp90 or yHsp90-K178R, and we determined the rate of CFTR protein decay in the presence of cycloheximide. We found CFTR stability to be significantly enhanced in yeast expressing yHsp90-K178R compared to wild-type cells (Figure 4G). A similar result was obtained in HEK293 cells transiently transfected with wild-type CFTR and with either wild-type hHsp90 α or hHsp90 α -K191R. The greater abundance of slower migrating CFTR in cells overexpressing hHsp90 α -K191R (Figure 4H) is consistent with previous observations that reduced Aha1 expression improves Hsp90-dependent CFTR maturation, presumably by allowing prolonged interaction of this difficult-to-fold client with Hsp90 (Wang et al., 2006). Thus, the enhanced CFTR chaperone efficiency of N domain lysine-mutated Hsp90 in both yeast and mammalian cell backgrounds likely reflects a physiological consequence of compromised Hsp90/Aha1 interaction.

Increased SUMOylation of the Hsp90 N Domain Inhibits Chaperone Function

Overexpression of SMT3 and SUMO-1 increased N domain SUMOylation of yHsp90 and hHsp90 α , respectively (Figures 2C and 2E). In contrast to lack of SUMOylation, increased SUMOylation of the Hsp90 N domain more generally affected Hsp90 chaperone activity. Overexpression of *SMT3-MYC*

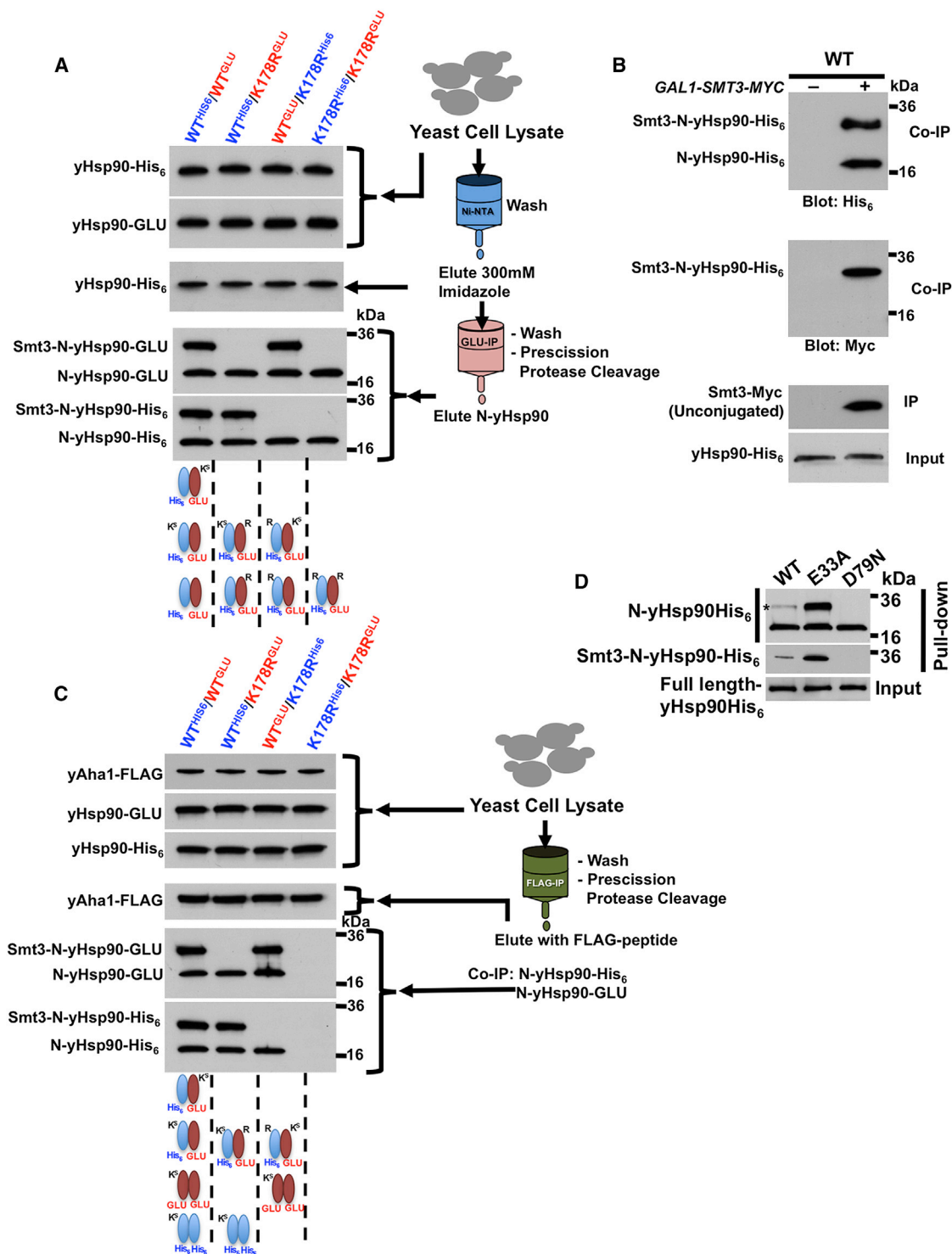


Figure 3. Aha1 Binds to Asymmetrically SUMOylated Hsp90

(A) Lysates from yeast cells overexpressing *GAL1-SMT3-MYC* and coexpressing wild-type yHsp90 (WT) or yHsp90-K178R (K178R) with either a His₆ or a GLU epitope tag (as indicated) were used to first precipitate His₆-yHsp90 with Ni-NTA agarose. Eluates were subsequently used to immunoprecipitate GLU epitope-tagged yHsp90. After PreScission Protease digestion, N domain SUMOylation was assessed by immunoblotting.

(B) Smt3-Myc was immunoprecipitated from wild-type yeast cells (WT) grown on either raffinose (–) or galactose (+). Immunoprecipitated Smt3-Myc and Smt3-Myc-modified proteins were treated with PreScission Protease, and N domain SUMOylation of Smt3-Myc coprecipitated Hsp90 proteins was assessed by immunoblotting.

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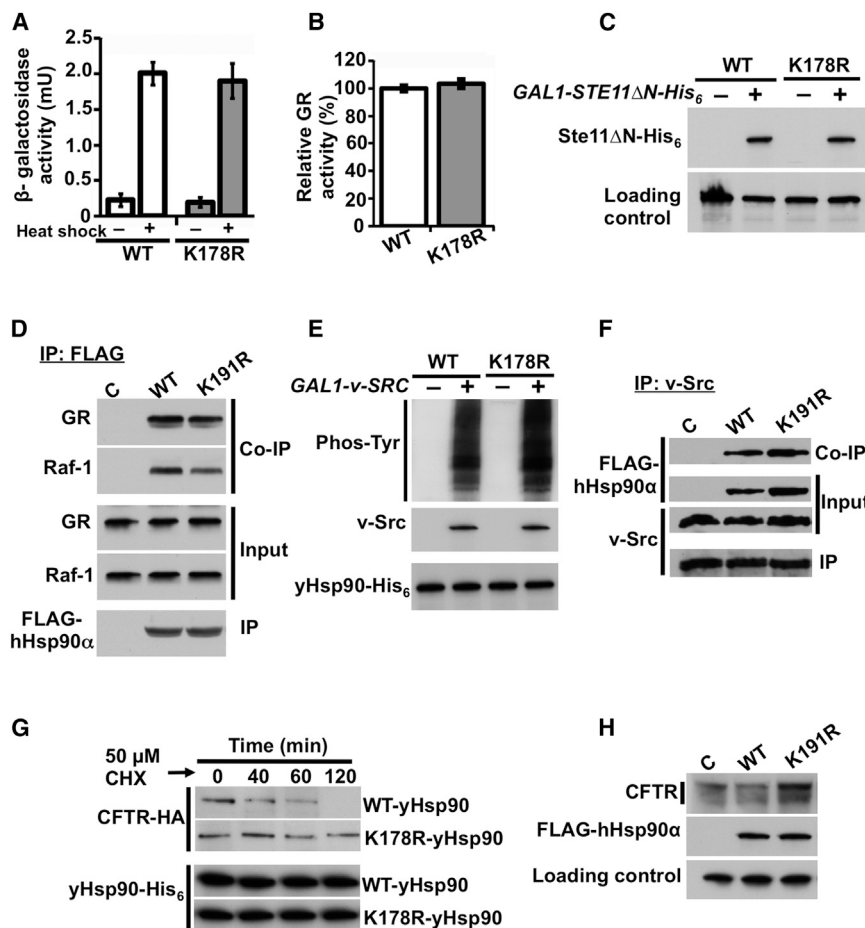


Figure 4. Lack of yHsp90 N Domain SUMOylation Has Little Effect on Most Hsp90 Clients

(A) Wild-type (WT) or yHsp90-K178R (K178R)-expressing yeast strains were heat shocked (40 min at 39°C). Heat shock response was measured in three independent experiments. All data represent mean \pm SD.

(B) GR-*lacZ* activity was assessed in the same yeast strains as above. Data are expressed as a percentage of the activity observed in wild-type cells and depicted as the mean \pm SD from values obtained in three independent experiments.

(C) Wild-type (WT) and yHsp90-K178R (K178R) yeast strains coexpressing Ste11 Δ N-His₆ under *GAL1* promoter were grown on raffinose (–) or galactose (+) media, and Ste11 Δ N-His₆ protein expression was examined by immunoblotting. α -tubulin was used as loading control.

(D) COS7 cells were transfected with indicated FLAG-Hsp90 α constructs. Hsp90 was immunoprecipitated with anti-FLAG agarose; coprecipitating (endogenous) GR and Raf-1 proteins were detected by immunoblotting.

(E) Wild-type (WT) and yHsp90-K178R (K178R) yeast containing *v-SRC* under *GAL1* promoter were grown on raffinose (–) or galactose (+) media. v-Src and total phosphotyrosine were detected by immunoblotting.

(F) NIH 3T3 cells stably expressing v-Src protein were transfected with empty plasmid (C), FLAG-hHsp90 α (WT), or FLAG-hHsp90 α -K191R (K191R) constructs. v-Src protein was immunoprecipitated, and associated FLAG-Hsp90 proteins were detected by immunoblotting.

(G) CFTR-HA was expressed in wild-type (WT) or yHsp90-K178R yeast; cells were treated with cycloheximide (CHX, 50 μ M) and harvested at the indicated time points. CFTR was analyzed by immunoblotting.

(H) HEK293 cells were transfected with wild-type CFTR and cotransfected with either wild-type FLAG-hHsp90 α (WT) or FLAG-hHsp90 α -K191R (K191R) constructs. After 24 hr, CFTR and FLAG-hHsp90 α were detected by immunoblotting. α -tubulin was used as loading control. See also Figure S3.

reduced CFTR stability in wild-type yeast, but not in yeast expressing yHsp90-K178R (Figures 5A and S4). Overexpression of *SUMO-1* in HEK293 cells cotransfected with CFTR and FLAG-tagged hHsp90 α proteins had a similar effect (Figure 5B).

Likewise, we found that *SMT3-MYC* overexpression in wild-type yeast led to both reduced v-Src expression and v-Src-mediated protein tyrosine phosphorylation (Figure 5C) as well as a 70% reduction in GR activity (Figure 5D), but this was not seen in yeast expressing yHsp90-K178R.

Finally, we studied the effect of SUMOylation on Hsf1 transcriptional activity. It is generally accepted that Hsp90 interaction suppresses Hsf1 activity (Zou et al., 1998), and compromised Hsp90 chaperone function, due to mutation or pharmacologic inhibition, leads to induction of Hsf1 activity in yeast even in the absence of heat shock (Hjorth-Sørensen et al., 2001). *SMT3-MYC* overexpression caused a heat shock

response in wild-type yeast, but not in yeast expressing yHsp90-K178R (Figure 5E). Similarly, we observed that the Hsp90 inhibitors geldanamycin (GA) and radicicol (RD) failed to induce a heat shock response in yHsp90-K178R-expressing yeast (Figure 5F). Importantly, mutation of K178 did not affect the heat shock response induced by elevated temperature (Figure 4A). These data indicate that N domain SUMOylation differentially affects the heat shock transcriptional response to elevated temperature and Hsp90 inhibitors, and they suggest possible similarities between deregulated Hsp90 SUMOylation and pharmacologic inhibition of the chaperone.

N Domain SUMOylation Sensitizes Cells to Hsp90 Inhibition

To explore the possible impact of Hsp90 SUMOylation on drug sensitivity, we first examined effects of GA and RD on

(C) Yeast cells in (A) coexpressing yAha1-FLAG under native promoter were used to immunoprecipitate yAha1 with anti-FLAG agarose. N domains from coprecipitated Hsp90 were then isolated by PreScission Protease digestion and analyzed by immunoblotting.

(D) Wild-type yHsp90-His₆ (WT), yHsp90-E33A-His₆ (E33A), or yHsp90-D79N-His₆ (D79N) constructs were expressed in yeast cells containing endogenous yHsp90. After Ni-NTA agarose pull-down and PreScission Protease digestion, N domain SUMOylation was assessed by immunoblotting.

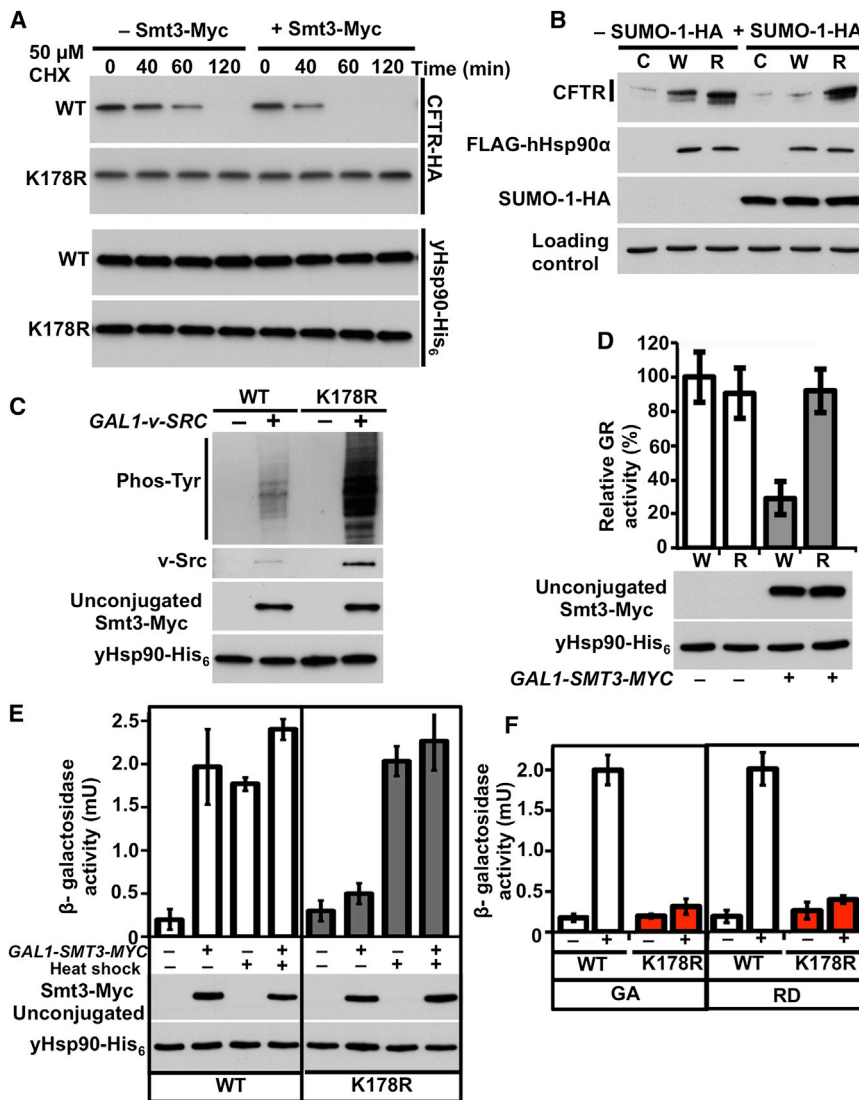


Figure 5. Increased Hsp90 N Domain SUMOylation Affects Chaperone Function

(A) Wild-type (WT) or yHsp90-K178R (K178R) yeast cells coexpressing CFTR-HA and GAL1-SMT3-MYC were grown on raffinose (–) or galactose (+) media, treated with cycloheximide (CHX, 50 μM), and harvested at the indicated time points. CFTR was visualized by immunoblotting.

(B) HEK293 cells were transfected with wild-type CFTR and cotransfected with empty plasmid (C), wild-type FLAG-hHsp90α (W), or FLAG-hHsp90α-K191R (R), with (+) or without (–) cotransfection of SUMO-1-HA. After 24 hr, CFTR, FLAG-hHsp90α, and SUMO-1-HA were detected by immunoblotting. α-tubulin was used as loading control.

(C) Wild-type (WT) and yHsp90-K178R (K178R)-expressing yeast containing GAL1-v-SRC and GAL1-SMT3-MYC were grown on raffinose (–) or galactose (+) media. v-Src, total phosphotyrosine, and Smt3-Myc were detected by immunoblotting. (D) GR-lacZ activity was assessed in wild-type (W) and yHsp90-K178R (R) yeast cells that also expressed GAL1-SMT3-MYC. Cells were grown on raffinose (–) or galactose (+) media. Data are expressed as a percentage of the activity observed in wild-type cells and depicted as the mean ± SD of three independent experiments.

(E) Heat shock element (HSE)-lacZ reporter was used to monitor the heat shock response in yeast expressing wild-type yHsp90 (WT) or yHsp90-K178R (K178R); yeast also expressed GAL1-SMT3-MYC. Cells were grown on raffinose (–) or galactose (+) media and heat shocked (40 min at 39°C). All data represent mean ± SD of three independent experiments.

(F) Wild-type yHsp90 (WT) and yHsp90-K178R (K178R) cells were treated with GA (+; 50 μM, 1 hr) or RD (+; 30 μM, 1 hr). Heat shock response was measured from three independent experiments. All data represent mean ± SD. See also Figure S4.

steady-state SUMOylation of K178. Unlike the response to α factor and heat shock (Figure S3A), N domain SUMOylation increased following exposure to GA or RD (Figure 6A). Since these drugs did not affect endogenous Smt3 expression, we reasoned that they might preferentially interact with, and conformationally trap, N domain SUMOylated Hsp90. We tested this possibility by overexpressing SMT3-MYC in wild-type yHsp90-expressing and yHsp90-K178R-expressing cells and using biotinylated Hsp90 inhibitor ganetespib and streptavidin beads to affinity purify yHsp90 proteins from cell lysates. We found that SMT3 overexpression clearly enhanced ganetespib recognition of wild-type yHsp90 but had little effect on recognition of yHsp90-K178R (Figure 6B).

Based on these observations, we asked whether increased N domain SUMOylation sensitized yeast cells to Hsp90 inhibition. Although we saw no difference in drug sensitivity between cells expressing wild-type yHsp90 or yHsp90-K178R under normal conditions (Figure 6C, “C” rows), after SMT3 overex-

pression cells harboring wild-type yHsp90 displayed greater sensitivity to a panel of four N domain inhibitors compared to yeast expressing yHsp90-K178R (Figure 6C, “GAL-SMT3” rows). It is notable that, at higher drug concentrations, lack of SUMOylation does not confer resistance to Hsp90 inhibitors (Figure S5A).

We carried out similar experiments in mammalian cells. Using immortalized NIH 3T3 mouse fibroblasts transiently transfected with plasmids encoding hemagglutinin (HA)-tagged SUMO-1, SUMO-2, or SUMO-3 proteins, we confirmed that only SUMO-1 overexpression sensitized the cells to ganetespib, as evidenced by induction of the proapoptotic markers: cleaved caspase and cleaved poly (ADP-ribose) polymerase (PARP) (Figure 6D). Likewise, Hsp90 N domain SUMOylation was detectable only in SUMO-1-transfected cells. It is notable that overexpression of SUMO-1 in the absence of Hsp90 inhibitor did not induce apoptotic markers in NIH 3T3 cells (Figure 6D, right panel).

Cancer cells generally display greater sensitivity toward Hsp90 inhibitors than do their nontumorigenic counterparts (Bisht et al., 2003). Therefore, we asked whether Hsp90 was SUMOylated to a greater degree in NIH 3T3 cells stably transformed with either v-Src or mutated (constitutively active) MET (MET-Y1428H) to a degree greater than that of parental NIH 3T3 cells and whether steady-state Hsp90 N domain SUMOylation intensity correlated with sensitivity to ganetespib. Indeed, both transformed cell lines displayed increased Hsp90 N domain SUMOylation compared to nontransformed cells (Figure 6E), and oncogenic transformation rendered the cells more sensitive to ganetespib, as evidenced by a greater abundance of apoptotic markers (Figure 6F).

These findings suggest that Hsp90 inhibitors and Aha1 compete for binding to SUMOylated Hsp90. To test this possibility, we overexpressed *SMT3-MYC* in yeast cells expressing wild-type yHsp90 and then treated the cells with GA. Interaction of yHsp90 and yAha1 was examined by Hsp90 pull-down and western blot. Although *SMT3-MYC* overexpression increased yHsp90 interaction with yAha1, association of the cochaperone with Hsp90 was not detectable in cells treated with GA prior to lysis (Figure S5B). Finally, we showed earlier (Figure 3D) that yHsp90-E33A (which binds ATP but does not hydrolyze it) is SUMOylated markedly more than wild-type yHsp90, and the equivalent mutation in hHsp90 α -E47A promotes increased steady-state interaction with Aha1 in cells (Xu et al., 2012). However, GA-affinity bead pull-down of hHsp90 α -E47A was less efficient than that of the wild-type protein, and Aha1 was not present in either pull-down (Figure S5C), consistent with the hypothesis that Hsp90 inhibitors and Aha1 cannot interact concurrently with N domain SUMOylated Hsp90.

DISCUSSION

In this study, we identified SUMOylation of a conserved lysine residue in the N domains of both yeast (K178) and human (K191) Hsp90. Mutation of this residue abrogated N domain SUMOylation and prevented detectable intracellular interaction with the ATPase-stimulating cochaperone Aha1. Based on *AHA1* deletion studies in yeast, Aha1/Hsp90 interaction is considered to be important for the chaperoning of Hsp90 clients, including those examined in this study (Holmes et al., 2008; Lotz et al., 2003; Panaretou et al., 2002). However, lack of detectable Aha1 interaction with yHsp90-K178R engendered observable effects only with the difficult-to-fold Hsp90 client CFTR, consistent with published reports demonstrating improved Hsp90-dependent CFTR folding in cells with reduced Aha1 expression (Koulov et al., 2010; Wang et al., 2006). Our data suggest that reduced Aha1/Hsp90 interaction per se does not phenocopy *yaha1 Δ* , implying that Aha1, like the cochaperones Sba1^{P23} and Cdc37^{P50} (Echtenkamp et al., 2011; Kimura et al., 1997), may have additional activities in cells that are independent of (but perhaps function in parallel with) its role in modulating Hsp90 activity (Sun et al., 2012).

In yeast, Aha1 is expressed at a concentration that is 10-fold lower than that of Hsp90 (Ghaemmamghami et al., 2003). Thus, although bacterially purified yAha1 binds to wild-type yHsp90 and yHsp90-K178R proteins with comparable affinity, it is

reasonable to assume that, in cells, certain Hsp90 PTMs modulate Aha1 recruitment to active Hsp90 chaperone complexes. Indeed, phosphorylation and acetylation of specific sites on Hsp90 have been shown previously to affect interaction with Aha1 (Mollapour et al., 2010, 2011a; Retzlaff et al., 2010; Scroggins et al., 2007). Our current data suggest that N domain SUMOylation is a PTM able to initiate recruitment of Aha1 to Hsp90 in both yeast and human cells. One molecule of Aha1 is sufficient to maximally stimulate Hsp90 ATPase activity (Panaretou et al., 2002; Soroka et al., 2012; Xu et al., 2012). The Aha1 N domain is thought to interact first with the middle domain of one Hsp90 protomer, after which the Aha1 C domain associates with the N domain of the opposing protomer (or with a hydrophobic groove formed by transient, ATP-dependent dimerization of both Hsp90 N domains) (Koulov et al., 2010; Meyer et al., 2003; Retzlaff et al., 2010). Although, as of yet, we have not uncovered the mechanism by which Hsp90 N domain SUMOylation facilitates Aha1 interaction, the asymmetry of this PTM (and its conservation in yeast and human cells) is consistent with a role in promoting the asymmetric interaction of Aha1 and Hsp90 in cells.

Increased SUMOylation of the Hsp90 N domain, although predicted to accelerate the rate of chaperone cycling, functionally compromised several Hsp90 clients, including v-Src, GR, and CFTR. These data are consistent with previous studies examining the impact of other Hsp90 modifications that stimulate ATP hydrolysis. Thus, the yHsp90 mutant T221 demonstrates both enhanced ATPase activity and ATP-dependent N domain dimerization (Prodromou et al., 2000) while possessing reduced ability to chaperone both GR and v-Src (Nathan and Lindquist, 1995). Similarly, although covalent N-terminal fusion of constitutively dimerized coiled-coil domains to yHsp90 enforces N domain proximity and significantly increases basal ATPase activity and yAha1 binding affinity, coiled-coil yHsp90 chaperones GR and v-Src less efficiently than does wild-type yHsp90 (Pullen and Bolon, 2011).

We explored the temporal relationship between N domain SUMOylation, ATP binding, and ATP hydrolysis with the help of two yHsp90 point mutants. yHsp90-E33A efficiently binds, but does not hydrolyze, ATP. Compared to wild-type yHsp90, this mutant was markedly more SUMOylated at steady-state. In contrast, yHsp90-D79N does not bind ATP and was not detectably SUMOylated. The simplest model that is consistent with these data suggests that ATP binding is a prerequisite for N domain SUMOylation. Although we cannot absolutely rule out the possibility that SUMOylation may also stabilize an ATP-bound Hsp90 conformation, the fact that SUMOylation of yHsp90-D79N was not observed reduces the likelihood of this hypothesis.

Surprisingly, ATP-competitive Hsp90 inhibitors increased steady-state N domain SUMOylation and readily recognized wild-type, but not nonSUMOylatable, yHsp90 in protein lysates of yeast overexpressing *SMT3*. Since association of Aha1 and these inhibitors with Hsp90 is mutually exclusive (Figure S5B), our data suggest that inhibitors preferentially bind to SUMOylated Hsp90 by displacing previously bound ATP prior to lid closure and Aha1-facilitated Hsp90 commitment to N domain dimerization and ATP hydrolysis, by which point

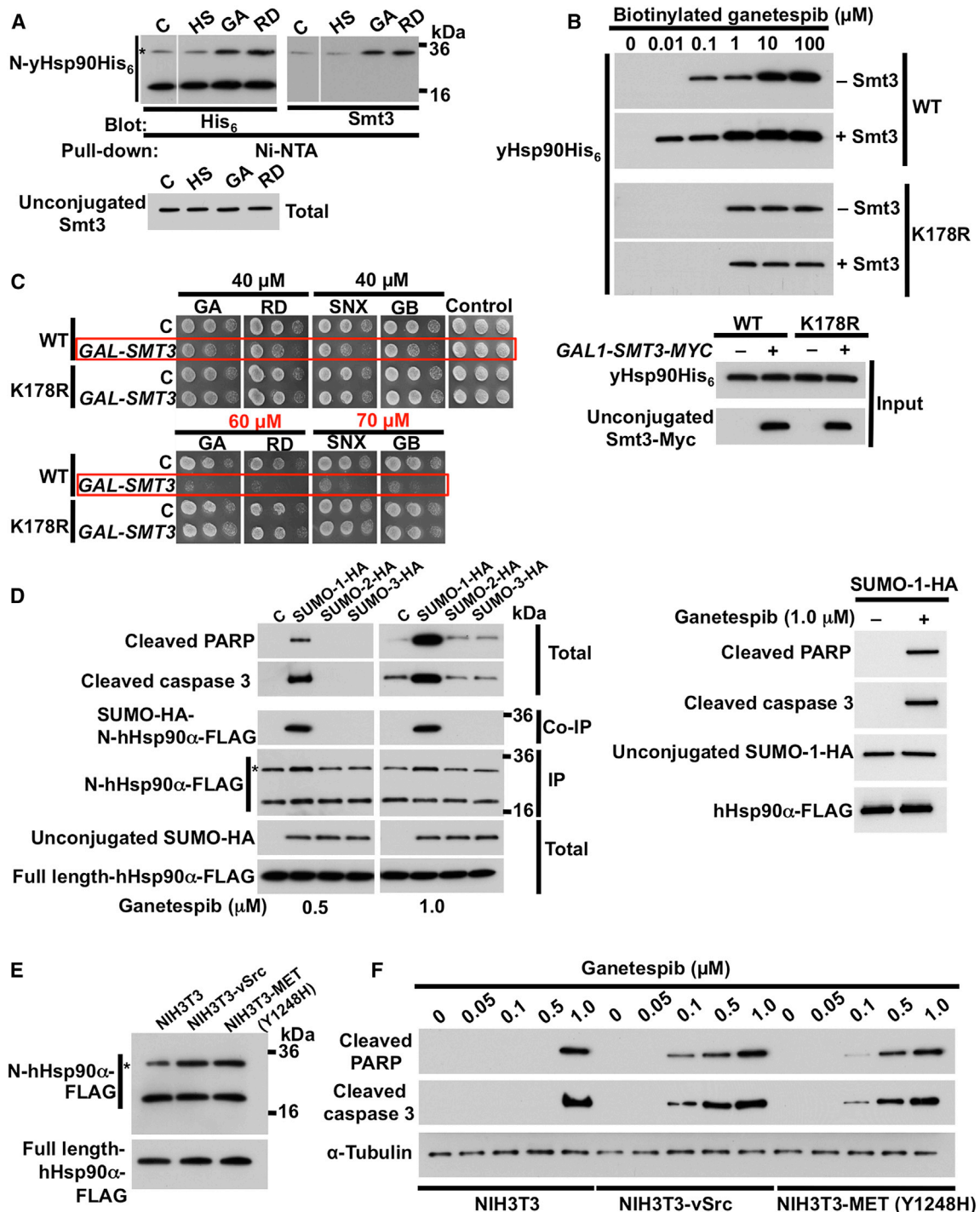


Figure 6. Increased Hsp90 N Domain SUMOylation Sensitizes Cells to Hsp90 Inhibitors

(A) The effects of heat shock (HS) (40 min at 39°C), GA (50 μM, 1 hr), or RD (30 μM, 1 hr) on SUMOylation of yHsp90-K178 (*) were monitored by immunoblotting with anti-hexahistidine. Unconjugated Smt3 was detected by anti-Smt3.

(B) Yeast cells expressing wild-type yHsp90 (WT) or yHsp90-K178R (K178R), and harboring GAL1-SMT3-MYC, were grown on galactose. Hsp90 was isolated from yeast lysates by incubating with indicated amounts of biotinylated ganetespi followed by streptavidin agarose beads and detected by immunoblotting with anti-His₆.

(C) Wild-type or K178R-yHsp90-expressing yeast harboring GAL1-SMT3-MYC was grown on galactose media for 4 hr. A 1:10 dilution series of 10⁷ cells/ml were spotted on YPDA agar containing indicated concentrations of the Hsp90 inhibitors GA, RD, SNX (SNX2112), or GB (ganetespi). Plates were incubated at 28°C for 4 days.

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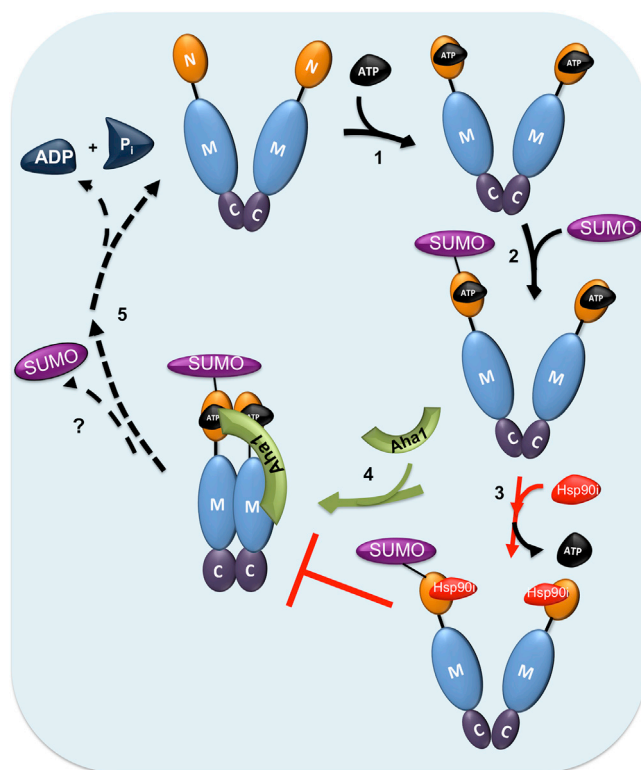


Figure 7. Hsp90 SUMOylation Facilitates Aha1 Association but Also Enhances Binding of ATP-Competitive Inhibitors

(1) The Hsp90 chaperone cycle is initiated by ATP binding to N domain(s). (2) This promotes asymmetric N domain SUMOylation. (3) Hsp90 inhibitors preferentially bind to SUMOylated Hsp90 by replacing ATP in the nucleotide binding pocket. (4) In the absence of N domain inhibitors, asymmetric N domain SUMOylation facilitates Aha1 association, accelerating the ATPase-driven Hsp90 chaperone cycle. (5) SUMOylation is dynamic, and Hsp90 deSUMOylation is predicted to occur prior to initiation of a new chaperone cycle.

conformational changes have rendered the ATP pocket inaccessible to these drugs. Drug binding to Hsp90 prevents N domain dimerization and Aha1 recruitment, trapping SUMOylated Hsp90 in an “open” conformation, unable to proceed further in the chaperone cycle (Figure 7). This model is consistent with a previous report that Aha1 knockdown sensitizes cancer cells to Hsp90 inhibitors (Holmes et al., 2008) and with our finding that the hydrolysis-incompetent yHsp90-E33A, which is trapped in an ATP-bound, lid-closed conformation, is SUMOylated to a degree greater than that of wild-type yHsp90 yet is poorly recognized by Hsp90 inhibitor (Figure S5C). Perhaps unexpectedly, this model predicts that Hsp90 inhibitors do not bind to all N domain undimerized (open) Hsp90 conformations with equal

affinity but can distinguish ATP-bound Hsp90 (prior to lid closure) from the ATP-unbound chaperone.

Finally, since Hsp90 inhibitors have binding constants much higher than those of ATP, this model predicts that drug trapping of SUMOylated Hsp90 preferentially removes a fraction of the actively cycling chaperone pool. Our observation that the intensity of Hsp90 N domain SUMOylation increases with cellular transformation is consistent with this proposal and provides mechanistic insight that helps clarify an observation made some years ago by Kamal et al. (2003), experimentally supported by subsequent studies but still not satisfactorily explained, that Hsp90 isolated from tumor cells simultaneously demonstrates both ATPase activity and affinity for Hsp90 inhibitors greater than those of Hsp90 isolated from nontransformed cells. The tumor cell selectivity of these drugs thus may reflect a greater dependence of cancer cells (relative to nontransformed cells) on the constitutive engagement of a significant fraction of Hsp90 in the chaperone cycle.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Growth Media

Yeast strain pp30 (*MAT a*, *trp1-289*, *leu2-3,112*, *his3-200*, *ura3-52*, *ade2-101*, *lys2-801*, *hsc82KANMX4*, *hsp82KANMX4*) was used in this study (Panaretou et al., 1998). Plasmids were constructed as described previously (Mollapour et al., 2011b). Detailed procedures, a list of primers (Table S1), and media conditions for both yeast and mammalian cells are provided in the Supplemental Information.

Protein Extraction, Immunoprecipitation, PreScission Protease Cleavage, and Immunoblotting

Total protein extracts were prepared and analyzed by western blotting as described previously (Mollapour et al., 2010), with the exception that N-ethylmaleimide (NEM, 20 mM) was included in the protein lysis buffer. Detailed methods for protein precipitations, cleavage by PreScission Protease, and detection by western blotting for both yeast and mammalian systems are presented in the Supplemental Experimental Procedures.

Assays for Hsp90 Client Activity

v-Src induction and activation were analyzed as described previously (Mollapour et al., 2011b). Expressed v-Src protein was detected with EC10 mouse antibody (Millipore) and v-Src activity with 4G10 mouse anti-phosphotyrosine (Millipore). GR assay was performed as described previously (Garabedian and Yamamoto, 1992), as was measurement of HSE-LacZ expression (Hjorth-Sørensen et al., 2001). Ste11ΔN induction was analyzed as described previously (Flom et al., 2008; Louvion et al., 1998). Ste11ΔN plasmid is a gift of Jill Johnson. Additional details are found in the Supplemental Experimental Procedures.

Isothermal Titration Calorimetry and K_D Determinations

Isothermal titration calorimetry (ITC) and K_D determinations were performed as described previously (Prodromou et al., 2000). Additional information can be found in the Supplemental Experimental Procedures.

(D) NIH 3T3 cells were transfected with empty plasmid (C), SUMO-1-HA, SUMO-2-HA, or SUMO-3-HA. After 24 hr, cells were treated with either 0.5 μ M or 1.0 μ M ganetespib for an additional 6 hr. SUMO-1, SUMO-2, and SUMO-3, hHsp90 α N domain SUMOylation, cleaved PARP, and cleaved caspase-3 were detected by immunoblotting.

(E) Parental NIH 3T3 cells and NIH 3T3 stably expressing v-Src or constitutively active MET (Y1248H) mutant were transfected with wild-type FLAG-hHsp90 α (containing a PreScission cleavage site). After 24 hr, N domain SUMOylation (*) of FLAG-hHsp90 α was assessed by immunoblotting with anti-FLAG following PreScission Protease digestion.

(F) Parental NIH 3T3 cells and NIH 3T3 cells stably expressing v-Src or constitutively activated MET (Y1248H) mutant were treated with indicated amounts of ganetespib for 6 hr. Cleaved PARP and cleaved caspase-3 were detected by immunoblotting. α -tubulin was used as a loading control. See also Figure S5.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2013.12.007>.

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